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High performance liquid chromatography/ion-trap mass spectrometry for separation and simultaneous determination of ethynylestradiol, gestodene, levonorgestrel, cyproterone acetate and desogestrel

David Matějíček, Vlastimil Kubáň *

Department of Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, Zemědělská 1, Brno, CZ 613 00, Czech Republic

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Abstract

A fast and highly sensitive high performance liquid chromatographic/ion-trap mass spectrometric method (LC/MS) has been developed for simultaneous determination of ethynylestradiol (EE2), gestodene (GES), levonorgestrel (LNG), cyproterone acetate (CPA) and desogestrel (DES). Among three types of sorbents tested (C8, C18 and phenyl) from two suppliers, the best separation was achieved on reverse phase Zorbax SB-Phenyl column using aqueous methanol as a mobile phase. A linear gradient profile from 70 up to 100% (v/v) in 7th min, kept constant at 100% up to 10th min and followed by a negative gradient to 70% of methanol up to 12th min was used for elution. Applicability of electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) and influence of the mobile phase composition, its flow rate, capillary/vaporizer temperature of API source and in-source fragmentor voltage ionization are discussed. The on-column limits of quantification (10 S/N) were 300 pg of EE2, 14 pg of GES and LNG, 4 pg of CPA and 960 pg of DES per injection (1 μL) using APCI with data collection in selected ion monitoring (SIM) mode. The analytical performance of the method was evaluated using the determination of EE2, GES, LNG, CPA and DES in contraceptives and river water samples.

Keywords: Liquid chromatography; Ion-trap mass spectrometry; Electrospray ionization; Atmospheric pressure chemical ionization; Ethynylestradiol; Gestodene; Levonorgestrel; Cyproterone acetate; Desogestrel

1. Introduction

Chemicals that disrupt endocrine functions have been found in the environment. They are often linked to adverse effects on the reproductive system in wildlife and humans. Many reasonable suspicions have been raised concerning the environmental impact of synthetic contraceptive chemicals as well as the endogenous estrogens excreted by humans and animals. More stable estrogens, such as ethynylestradiol (EE2) and progestogens (or progestins), such as levonorgestrel (LNG), gestodene (GES), cyproterone acetate (CPA) and desogestrel (DES) are used more frequently for medical purposes (e.g. contraception, treatment of prostate and breast cancer, treatment of infertility).

Desogestrel and gestodene represent the latest generation of progestins and begin to replace levonorgestrel in oral contraceptives. The increasing use of incoming substances together with their high physiological activity and higher stability may lead to their ubiquitous occurrence in the environment.

The estrogens content in medical preparations, used in the management of menstrual and menopausal disorders as well as for contraception, is usually in a daily range 20–50 μg. As for the progestin content, it varies depending on the type of contraceptive. Thus, in combined oral formulations the progestin content is in a daily range 0.05–2 mg whereas it is lower in progestin-only contraceptives [1,2].

For many years, immunoassay methods have been the most sensitive analytical procedures available for the determination of estrogens in biological samples. These methods are sensitive, but are time consuming and prone to cross reactivity by endogenous steroids, co-administrated steroids and their metabolites. Gas

* Corresponding author. Tel.: +420 545133285; fax: +420 545212044.
E-mail address: kuban@mendelu.cz (V. Kubáň).

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chromatographic/mass spectrometric (GC/MS) methods typically employ some type of extraction (liquid–liquid or solid phase), and one or multiple steps of derivatization [2,4,5].

Recently, liquid chromatography coupled with electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) or atmospheric pressure photospray ionization-tandem mass spectrometry has been applied for the quantitative analysis of estrogens in environmental [3–9] and biological samples [10–13]. Liquid chromatography with tandem mass spectrometric detection was demonstrated to be superior to immunoassay methods or GC/MS in terms of selectivity, sensitivity, simplicity and analytical throughput [1,2,4,5].

Ethynylestradiol together with levonorgestrel belong to the commonly investigated estrogens in water, effluents of wastewater treatment plants, sewage sludge, soils, sediments and biological samples such as plasma and urine. Many methods based on a combination of a single extraction step, eventual cleanup and analysis (e.g. for water sample, solid-phase extraction, followed by chromatographic separation with mass spectrometric detection) have been published [1–6,10,11,14–17].

The use of electrospray-tandem mass spectrometry (ESI-MS–MS) in negative ionization mode for ethynylestradiol and in positive ionization mode for levonorgestrel has become dominant technique of their determination. Reported limits of detection (LODs) varied from 0.08 to 10 ng L^{-1} (resp. ng kg^{-1}) of ethynylestradiol and 1 to 20 ng L^{-1}(resp. ng kg^{-1}) of levonorgestrel in dependence on sample matrix, method of sample preparation and type of mass spectrometer used. The lowest detection limits were achieved using a triple–quadrupole mass spectrometer. Only limited number of information concerning cyproterone acetate, desogestrel and gestodene mass spectrometric determination have been published [12,18–20].

The aim of this study was to evaluate a liquid chromatographic/ion-trap mass spectrometric method (LC/MS) for the simultaneous determination of cyproterone acetate, gestodene and desogestrel together with ethynylestradiol and levonorgestrel and to test the applicability of the proposed method for their determination (see Fig. 1 for their molecular structures) in pharmaceutical dosage forms and in real and spiked water samples.

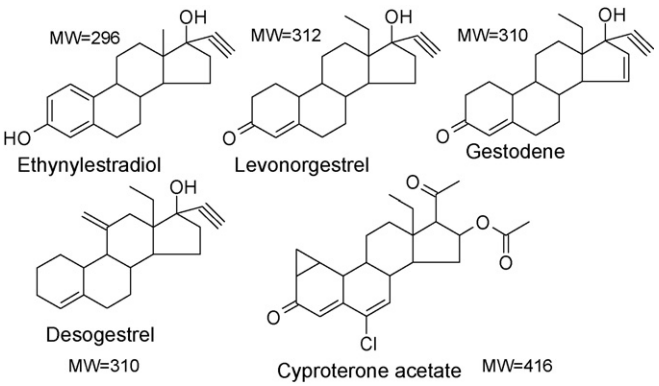

Fig. 1. Molecular structures of the investigated estrogen and progestins.

2. Experimental

2.1. Chemicals

Ethynylestradiol (EE2) was obtained from Riedel-de Haen (Seelze, Germany). *D*(−)-norgestrel (levonorgestrel, LNG) and cyproterone acetate (CPA) were purchased from Sigma–Aldrich (St. Louis, MA, USA). Gestodene (GES), desogestrel (DES) were obtained from Council of Europe, European Pharmacopoeia (Strasbourg, France). Stock standard solutions of each of the compounds ($c = 50\ \mu g\ mL^{-1}$) were prepared in methanol and stored in a refrigerator in darkness at 5 °C. The stability of the stock solutions of standards was controlled for 2 month and no change in concentration was observed. Working solutions were prepared daily by mixing and diluting the stock solutions with methanol.

The LC/MS Chromasolv® acetonitrile and methanol from Riedel-de Haen were used. Ammonium formiate (for MS), ammonium hydroxide solution (Trace Select Ultra) and formic acid (for MS) were purchased from Fluka Chemie (Buchs, Switzerland). De-mineralized water obtained by reverse osmosis using an AquaDem 02 (Aqua Osmotic, Tišnov, Czech Republic) was further purified using a MILLI-Q-RG (Millipore, Bedford, MA, USA).

2.2. High-performance liquid chromatography

An Agilent 1100 chromatographic system (Agilent, Waldbronn, Germany) equipped with a vacuum degasser, a quaternary pump, an autosampler, a column thermostat and a diode array detector was used. UV–vis spectra were automatically acquired for all peaks (range 190–400 nm, 2 nm steps). The system was coupled on-line to an ion-trap mass spectrometer Finnigan LCQ Advantage Max (San Jose, CA, USA). The XCalibur software (Version 1.4) controlled the whole liquid chromatographic/mass spectrometric system.

Following chromatographic columns were investigated: Zorbax SB-Phenyl (100 mm × 2.1 mm i.d., 3.5 μm particle size), Zorbax SB-Phenyl (75 mm × 4.6 mm i.d., 3.5 μm particle size), Zorbax Eclipse XDB C18 (50 mm × 2.1 mm i.d., 1.8 μm particle size), Zorbax Eclipse XDB C8 (50 mm × 2.1 mm i.d., 1.8 μm particle size), Zorbax Eclipse XDB C18 (50 mm × 4.6 mm i.d., 1.8 μm particle size), all products of Agilent (Palo Alto, CA, USA); Luna C8(2) (50 mm × 2.0 mm i.d., 3 μm particle size), Luna C18(2) (50 mm × 2.0 mm i.d., 3 μm particle size) and Luna Phenyl-hexyl (50 mm × 2.0 mm i.d., 3 μm particle size), all product of Phenomenex (Torrance, CA, USA).

The best results were obtained with Zorbax SB-Phenyl columns using an aqueous methanol as a mobile phase. A linear gradient profile from 70 up to 100% (v/v) of methanol in 7th min, kept constant at 100% of methanol up to 10th min and followed by a negative gradient to 70% of methanol up to 12th min was used for elution. The column was equilibrated prior to injection of each sample with the mobile phase containing 70% (v/v) of methanol for 5 min. The flow rate was 0.25 mL min^{-1} (2.1 mm columns) and 1.0 mL min^{-1} (4.6 mm columns), respectively. The column temperature was maintained at 35 °C. The

Table 1
ESI-MS and APCI-MS source optimized settings

Parameter	EE2	GES	LNG	CPA[a]	CPA[b]	DES
ESI mode						
Capillary temp. (°C)	375	375	375	375	375	375
Sheath/auxiliary gas flow rate (units)	24/3	26/5	23/4	23/5	12/3	20/10
Spray current (μA)	4.55	5.3	5.3	5.3	4.55	6
Capillary voltage (V)	−6	14	19	28	−16	20
Tube lens offset (V)	−30	25	30	30	−25	40
APCI mode						
Vaporizer temp. (°C)	350	350	350	350	325	375
Capillary temp. (°C)	150	150	150	150	150	150
Sheath/auxiliary gas flow rate (units)	40/18	65/26	72/36	40/27	36/5	60/30
Spray voltage (kV)	10	10	10	10	10	10
Capillary voltage (V)	−16	6	3	22	−40	6
Tube lens offset (V)	−55	34	40	55	−60	40
The most abundant ions (*m/z*)[c]	295/269	311/285	313/287	417/357	415/355	311/293,285
Ion transition (*m/z*)[d]	295 → 269	311 → 285	313 → 287	417 → 357	415 → 355	293 → 267
Relative collision energy (%)[e]	52	46	46	58	56	36

[a] Positive mode.
[b] Negative mode.
[c] Molecular/fragment ions used for quantification.
[d] Parent to product ion transitions used for the selected reaction monitoring (SRM).
[e] Relative collision energy values.

EE2 was monitored at λ = 280 nm, GES and LNG at λ = 244 nm, CYP at λ = 284 nm and DES at λ = 210 nm.

2.3. Mass spectrometry

The effluent from liquid chromatograph was directly introduced into the ion-trap mass spectrometer equipped with an electrospray ionization or atmospheric pressure chemical ionization source operated in positive (GES, LNG, CPA and DES) or negative (EE2, CPA) mode. The MS conditions were optimized for each of the tested compounds by direct injection of the solutions of the individual compounds at a concentration of 1 ng μL^{-1} standard solution (5 μL) into the 70% aqueous methanol at the flow rate 0.25 mL min^{-1} as the mobile phase. The chromatographic column was replaced with the PEEK restriction coil (0.03 mm × 300 mm). The relevant ion source settings for each compound are shown in Table 1 for electrospray and atmospheric pressure chemical ionization, respectively. The spectra were recorded from *m/z* 80 to 600. For the quantification, the MS data were acquired in selected ion monitoring (SIM) mode at *m/z* given in Table 1. Collision energy of source fragmentation was set to 10 eV for ESI and to 5 and 10 eV (desogestrel) for APCI source. The parent to product ion transitions used for the selected reaction monitoring (SRM) and the relative collision energy values are given in Table 1. Helium was used as the collision gas in the ion trap. The mass spectrometer operated at unit mass resolution.

2.4. Real sample preparation

2.4.1. Assay of estrogenic compounds in the contraceptive products

The following four pharmaceutical products were under assay: Minulet® (Wyeth-Lederle Pharma, Vienna, Austria) contains 30 μg of ethynylestradiol and 75 μg of gestodene per tablet; Minisiston® (Jenapharm, Jena, Germany) contains 30 μg of ethynylestradiol and 125 μg of levonorgestrel per tablet; Mercilon® (N.V. Organon, Oss, Netherland) contains 20 μg of ethynylestradiol and 150 μg of desogestrel per tablet and Diana35® (Shering AG, Berlin, Germany) contains 35 μg of ethynylestradiol and 2 mg of cyproterone acetate per tablet.

A single tablet was crushed in a porcelain mortar to a fine powder and transferred into 10 mL volumetric flask containing about 5 mL of methanol. This mixture was sonicated for 5 min, and then filled to the volume with methanol. The content of the flask was sonicated again for 10 min to complete dissolution of the drug. Acetonitrile and aqueous methanol or acetonitrile (1:1, v/v) were also tested as solvents. The final solution was filtered through a 0.45-μm PTFE membrane filter and 1 μL aliquots were injected into the LC/MS system operated in APCI mode. No internal standards were added. High content of cyproterone acetate in Diana35® drug required dilution of the extract prior to LC/MS analysis. Final concentration of EE2 in this solution was 500 pg μL^{-1} and concentration of cyproterone acetate was 25.5 ng μL^{-1}.

2.4.2. Assay of estrogenic compounds in spiked water samples

Ten water samples were collected in the Svratka river flowing Brno (Czech Republic), the town having population of about 400,000. The sites of collection were chosen randomly, upstream (5 pcs) and downstream (5 pcs) of wastewater treatment plant (WWPT), from Brno dam and tap water from the local supplier. Samples were filtered through a 0.45-μm PTFE membrane filter to remove solid particles, spiked with standard solution of selected estrogenic compounds to the concentration 100 pg μL^{-1} of ethynylestradiol, 10 pg μL^{-1} of gestodene, levonorgestrel, cyproterone acetate and 200 pg μL^{-1} of desogestrel

and then 3, 5 and 10 μL aliquots were injected into the LC/MS system operated in APCI mode.

3. Results and discussion

3.1. Liquid chromatography with UV detection

The type of the sorbent, the flow rate of the mobile phase and its composition with respect to the ratio of 'electrolyte' modifier and organic modifier are the main parameters responsible for obtaining best chromatographic separation.

As a first stage to the development of the chromatographic method, three types of sorbents (C8, C18 and phenyl) from two suppliers were investigated. The columns packed with phenyl sorbents gave the best results in terms of selectivity. Octyl- and octadecyl-sorbent showed poor resolution for ethynylestradiol and gestodene. These compounds were resolved with $R > 1.5$ after prolonging the duration of the separation and changing the gradient profile when the columns were packed with 1.8 μm particle size sorbents. Number of theoretical plates (N) calculated for gestodene and levonorgestrel were 4200 and 5600, respectively, when Zorbax SB-Phenyl (3.5 μm particle size) column was used. Number of the theoretical plates for gestodene and levonorgestrel was practically the same when Zorbax C8 and C18 columns (1.8 μm particle size) were used, but EE2 and GES remained unresolved. Chromatographic separations of the standards on different columns are shown in Fig. 2.

The gradient elution profile was optimized by using different ratios of acetonitrile to methanol, applying different modifiers (water, ammonium formiate at pH 3.0, 6.2 and 7.8 and formic acid), changing the flow rate of the mobile phase and the column oven temperature to achieve sufficient resolution of the tested compounds ($R > 1.5$) and shortest analysis time (less than 10 min). Gradient elution was required due to high hydrophobicity of desogestrel. All optimizations were performed with respect to mass spectrometric detection.

The best separation was obtained using a linear gradient of the mobile phase consisting of methanol and water. Changes in flow rate of the mobile phase and the temperature did not significantly improve the resolution. Similarly, changes of modifier and its pH did not affect the retention times and resolution. The concentration of modifiers in water was limited (5×10^{-3} mol L^{-1} at maximum) due to application of mass spectrometric detection. The best separation was achieved on reverse phase Zorbax SB-Phenyl column and using the abovementioned linear gradient profile of aqueous methanol as the mobile phase.

All compounds were well resolved with resolution $R > 2.0$. The peak symmetry ranged from 0.88 to 1.33. Calibration curves for all compounds were strictly linear. The limits of quantification (LOQs for S/N = 10) varied from 1.3 to 2.6 ng per injection. The repeatability (R.S.D.s 2.8–3.2%) was evaluated from the injection of five replicates of the standard solution in the concentration range 1(5)–50 ng. Appropriate UV spectra of individual

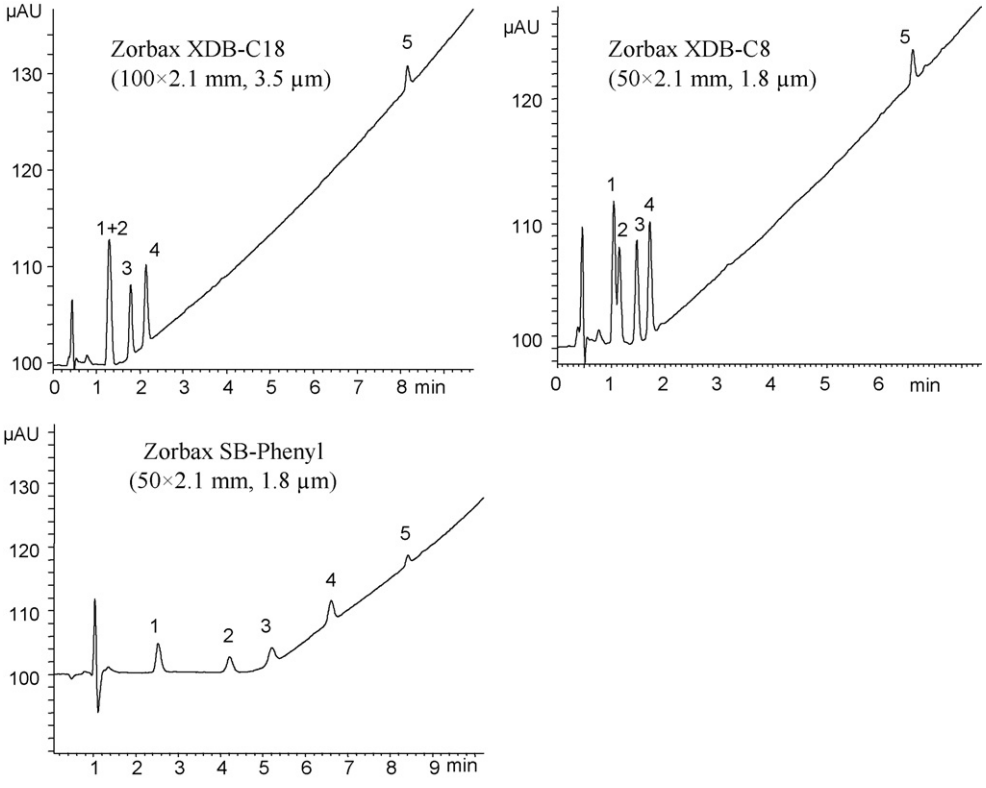

Fig. 2. The comparison of the separation of EE2, GES, LNG, CPA and DES standards on the Zorbax XDB-C8 and C18 (50 mm × 2.1 mm i.d., 1.8 μm particle size) and Zorbax SB-Phenyl (100 mm × 2.1 mm i.d., 3.5 μm particle size) columns using total DAD scan. The same chromatographic conditions (70% of methanol up to 100% of methanol (v/v) in 7 min, hold 2 min; $F = 0.25$ mL min^{-1}, $t = 35$ °C, 10 ng per injection) were applied. Key to peak identification: 1, ethynylestradiol; 2, gestodene; 3, levonorgestrel; 4, cyproterone acetate; 5, desogestrel.

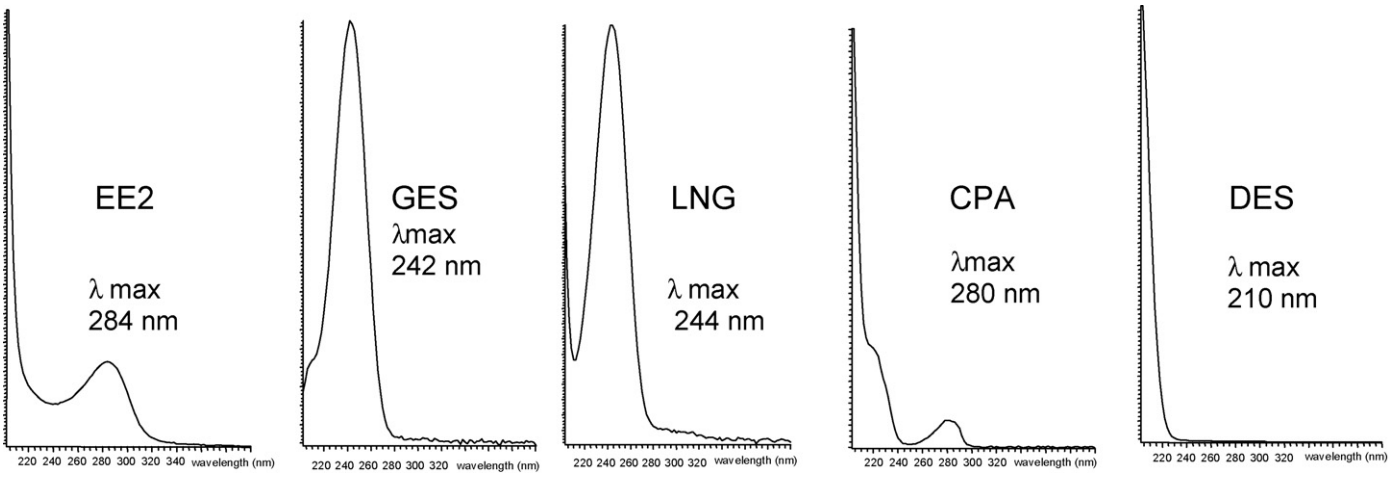

Fig. 3. UV–vis spectra of the individual compounds (the spectra acquired during the chromatographic run). Key to the compound identification: (1) EE2, ethynylestradiol; (2) GES, gestodene; (3) LNG, levonorgestrel; (4) CPA, cyproterone acetate; (5) DES, desogestrel.

compounds are shown in Fig. 3. For detailed information about separation and regression parameters see Table 2.

3.2. Liquid chromatography with MS detection

A LC/MS method was optimized with respect to individual parameters of both steps—the separation in the LC part and ionization and ions transport conditions in the MS part. The efficiency of ionization processes in reversed phase LC/MS was mainly influenced by electrolyte composition and temperature of the capillary, as well as vaporizer temperature for APCI. Similarly gas flow rate, spray voltage or current, capillary voltage, tube lens offset, multiple offset, lens voltage and multipole RF amplitude (optimized data not shown) were optimized to ensure maximal sensitivity of each compound.

APCI and ESI interfaces were evaluated for the determination of the selected estrogen—ethynylestradiol and progestins in both the positive and negative modes. EE2 was not detected with positive mode; similarly, gestodene, levonorgestrel and desogestrel were not detected in negative mode. Cyproterone acetate showed response in both the positive and the negative mode.

3.2.1. Mass spectrometric detection optimization

The effect of an organic solvent was investigated with methanol and acetonitrile by direct injection of the standard solutions. Approximately 40% better ionization of all tested compounds was achieved for methanol in comparison to acetonitrile. A rapid decrease of the ionization efficiency was observed when a mixture of water and acetonitrile was used in comparison to a mixture of water and methanol. No source fragmentation energy was used and ionization conditions were optimized for both organic solvents.

The influence of electrolyte composition on efficiency of ionization was tested for water, ammonium formiate with pH 3, 6.2 and 7.8 and formic acid, the most commonly used modifiers. Data given in Fig. 4 are shown for ESI mode, similar ones were obtained for APCI. No in-source fragmentation energy (CID) was used and ionization conditions were optimized for all solvents and tested electrolytes. In general, the aqueous methanol can be strongly recommended as the mobile phase for electrospray and atmospheric pressure chemical ionization of ethynylestradiol, gestodene, levonorgestrel, cyproterone acetate and desogestrel.

3.2.2. Effect of temperature

A proper setting of the capillary temperature for electrospray or vaporizer temperature in conjunction with a capillary temperature for APCI source represents one of the basic parameters affecting MS signal intensity. The optimized capillary temperature of ESI source for the tested compounds in positive

Table 2
Chromatographic separation with the UV detection using the Zorbax SB-Phenyl column (100 mm × 2.1 mm i.d., 3.5 μm particle size)

Peak no.	Compound	RT[a]	c (ng)[b]	r^2[c]	Symmetry	LOQ (ng)[d]	R.S.D.s (%)[e]
1	Ethynylestradiol (EE2)	2.53	5–200	0.9998	1.05	2.2	3.1
2	Gestodene (GES)	4.22	1–200	0.9999	0.88	1.5	2.8
3	Levonorgestrel (LNG)	5.21	1–200	0.9997	1.25	1.4	3.2
4	Cyproterone acetate (CPA)	6.61	1–200	0.9995	1.31	1.3	2.9
5	Desogestrel (DES)	8.42	5–200	0.9989	1.33	2.6	2.9

Injected volume was 1 μL.
[a] RT: The retention time in minutes, R.S.D.s from 0.16 to 1.1% ($n = 5$).
[b] Concentration range (in ng).
[c] Regression coefficients of the calibration curve for 40, 80, 120, 160 and 200 ng and the lowest concentration values.
[d] Limit of quantification equals to $10\,s_{bl}$, calculated for λ = 280 nm (EE2), λ = 244 nm (GES, LNG), λ = 284 nm (CPA) and λ = 210 nm (DES).
[e] $n = 5$, concentration level 5 ng μL^{-1} for EE2, DES and 1 ng μL^{-1} for GES, LNG, CPA.

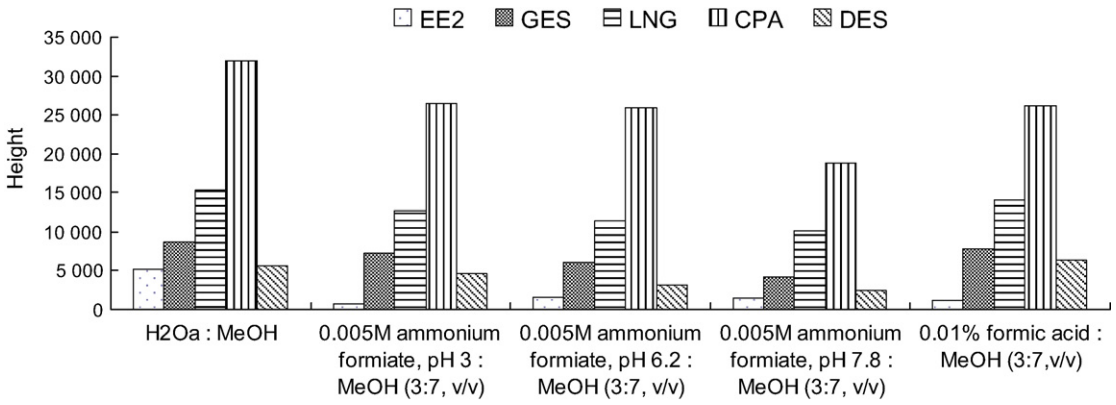

Fig. 4. The influence the mobile phase composition on the ionization efficiency (peak height) for the electrospray ionization mode using the flow injection analysis of 1 ng μL^{-1} of EE2, GES, LNG, CPA and DES. Injection volumes differed for the individual compounds. Key to the compound identification: EE2, ethynylestradiol; GES, gestodene; LNG, levonorgestrel; CPA, cyproterone acetate; DES, desogestrel.

mode was 350 °C, while in negative mode the temperature was 375 °C (see Fig. 5). More significant changes of the MS signal intensity were observed in the negative mode for ethynylestradiol.

The vaporizer temperature and capillary temperature was simultaneously optimized in the APCI mode. The same setting of the capillary temperature (150 °C) was found to be optimal for all the tested compounds.

On the other hand, the optimum for vaporizer temperature depends on the individual compounds and in the case of cyproterone acetate varied for the positive and the negative APCI mode. Nevertheless, the quick change of the vaporizer temperature (several seconds are needed for the change of the vaporizer temperature of 20 °C) allowed to set correct vaporizer temperature and thus to accomplish maximum sensitivity for all the tested compounds. The atmospheric pressure chemical ionization mode showed similar dependence on a proper setting of the vaporizer temperature as the electrospray source. The influence of the vaporizer temperature was more obvious in the negative than in the positive mode. The influence of the capillary temperature on the MS signal intensity in the APCI mode was

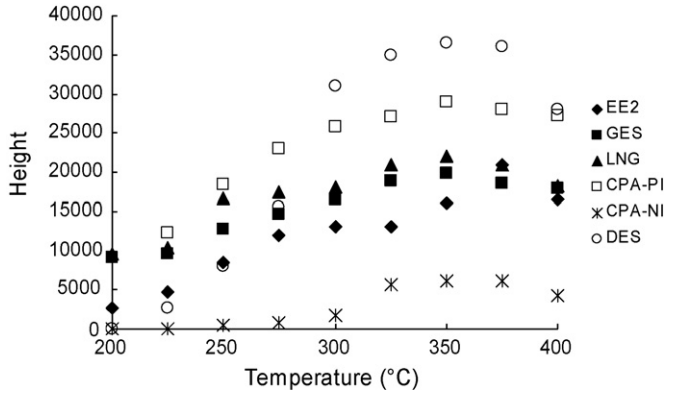

Fig. 5. The influence of the capillary temperature on the MS signals intensities in the ESI mode (other conditions see Fig. 4). Key to the compound identification: EE2, ethynylestradiol; GES, gestodene; LNG, levonorgestrel; CPA, cyproterone acetate; DES, desogestrel.

less serious than for the vaporizer temperature (data are not shown).

3.2.3. Flow variables

The effect of the flow rate on yield of ionization of both, ESI and APCI, modes was investigated by the direct injection of standard solutions (FIA/MS). In general, the flow rate of the mobile phase in the electrospray mode is recommended to be as low as possible, but the flow rate of the mobile phase up to 1 mL min^{-1} without splitting of the solvent prior to the MS detector is commonly used. On the other hand, the flow rate of the mobile phase flowing from the LC into the MS detector in the APCI mode is typically higher (between 0.2 and 2 mL min^{-1}). The influence of the flow rate of the solvent on the peak intensity was negligible in case of the APCI mode for flow rates between 0.2 and 1.5 mL min^{-1}. Only the sheath gas and auxiliary gas flow rates had to be adjusted depending on the flow rate of the mobile phase to ensure stability of the MS signal.

The influence of the mobile phase flow rate was significant in case of the ESI source, since any decrease of the flow rate caused a rise of the signal of the MS detector. The maximum peak heights for all tested compounds were achieved at the flow rate set to 0.1 mL min^{-1}. The flow rate of 0.25 mL min^{-1} is the good compromise for the used column with 2.1 mm inner diameter with regard to the duration of the chromatographic analysis and the intensity of the MS signal.

3.2.4. MS spectra

The fragmentation of the selected estrogenic compounds depended on the conditions of ionization and applied in-source fragmentor (CID) voltage. It was practically independent of the type of the ionization source. The same fragments were observed for the ESI and the APCI sources; only having different abundances. Spectra acquired in the APCI mode are given in Fig. 6.

The spectra for ethynylestradiol showed two most abundant ions, a molecular ion $(M-H)^-$ at *m/z* 295 and a fragment at *m/z* 269 corresponding to the elimination of C_2H_2 from the molecu-

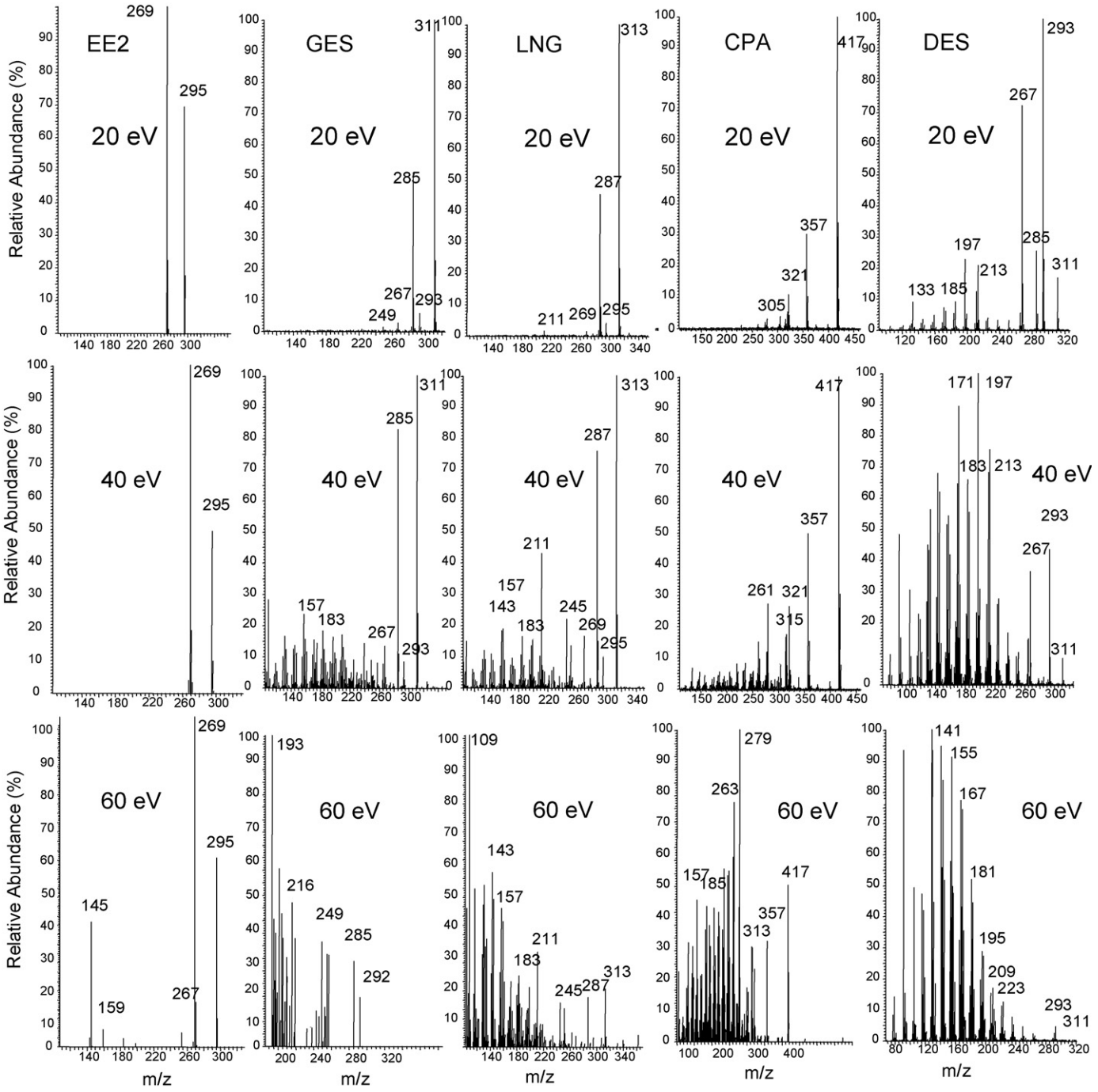

Fig. 6. The APCI-MS spectral characteristics of EE2, GES, LNG, CPA and DES with the fragmentor voltage ranging from 20 to 60 eV. Key to the compound identification: EE2, ethynylestradiol; GES, gestodene; LNG, levonorgestrel; CPA, cyproterone acetate; DES, desogestrel.

lar ion. The higher fragmentation energy led to a more intensive loss of C_2H_2 thereby change of the ratio of 295 and 269 ions. The fragmentation energy 60 eV led to the ring cleavages accompanied by H-rearrangement reactions giving fragment ions at *m/z* 159 and 145. They are believed to be products of losses of $C_9H_{12}O$ and $C_{10}H_{14}O$, respectively.

The molecular ion $(M+H)^+$ of gestodene at *m/z* 311 was obtained as the base peak even when 40 eV was applied. The fragmentation of the molecular ion led to the elimination of water (*m/z* 293) and liberation of the acetylene (*m/z* 285). The minor ion at *m/z* 267 corresponds to the loss of water together with the acetylene; ion at *m/z* 249 represents further loss of water molecule. The cleavage of the ring led to the ion at *m/z* 183 and 157 related to the fragmentation of $C_8H_{16}O$ and $C_{10}H_{18}O$ from the ring system, respectively.

Major ions of levonorgestrel spectra were at *m/z* 313, which corresponds to the molecular $(M+H)^+$ ion, and at *m/z* 287, which corresponds to the loss of acetylene. The similarity of

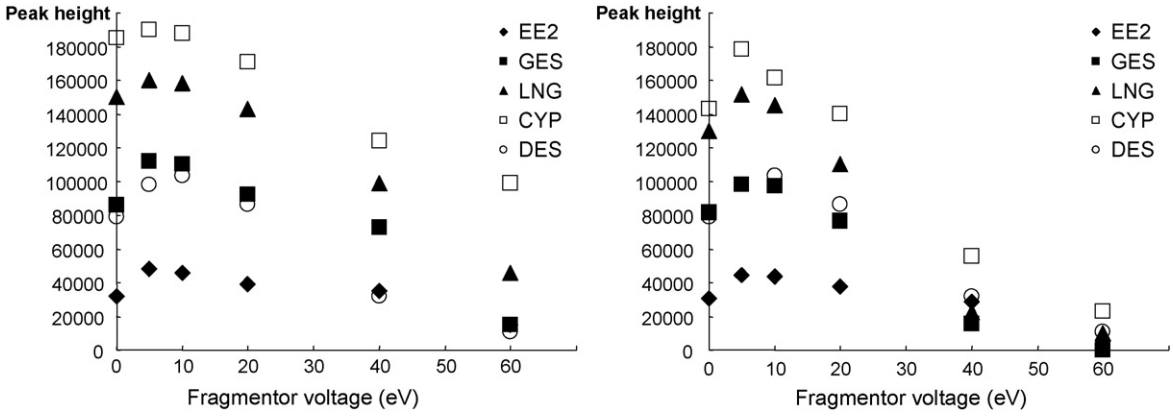

Fig. 7. The influence of the applied fragmentor voltage on the intensity of the MS signal. The peak height at A corresponds to the total ion current and at B corresponds to the ions selected for the SIM mode. Key to the compound identification: (1) EE2, ethynylestradiol; (2) GES, gestodene; (3) LNG, levonorgestrel; (4) CPA, cyproterone acetate; (5) DES, desogestrel.

the structures of levonorgestrel with gestodene led to similar abundance of the fragment ions at $m/z+2$ mass units (compare $311 \rightarrow 313$, $293 \rightarrow 295$, $285 \rightarrow 287$, and $267 \rightarrow 269$) and the same ions at m/z 183 and 157 that corresponds to the losses of $C_8H_{18}O$ and $C_{10}H_{20}O$ related to the D-steroidal ring, respectively. An ion at m/z 245 that corresponds to the steroidal ring without substituents was observed only when 40 eV was applied on fragmentor.

The most abundant ion in the cyproterone acetate spectrum was the molecular $(M+H)^+$ ion at m/z 417. The fragment at m/z 357 corresponds to the loss of CH_3COOH and m/z 321 to the loss of HCl. Ions at m/z 305 and 279 represent a step-by-step elimination of the acetyl group bonded on the C_{17} carbon. The fragmentor energy over 40 eV led to the cleavage of the steroidal ring. The spectra of cyproterone acetate collected in the negative mode show a molecular ion $(M-H)^-$ at m/z 415 followed by a fragment at m/z 355 that corresponds to the loss of acetic acid (the spectra are not shown).

The spectra of desogestrel express low stability of the molecular ion at m/z 311 under the given ESI as well as APCI ionization conditions. The relative instability of desogestrel led to the step-by-step elimination of the molecule of water or acetylene giving the fragments at m/z 293 or 285, elimination of both particles led to the fragment at m/z 269. The higher fragmentation energy led to the multiple cleavage of the steroidal ring accompanied by H-rearrangement reactions.

The product ion spectra (not shown) of the selected estrogenic compounds were strongly affected by the applied normalized collision energy and showed the same fragments as are given in Fig. 6. Application of the collision energy in the range 35–60% caused only the cleavage of the substituents in the form of water, acetylene, etc. The higher collision energy caused the more specific cleavage of the ring giving the fragment ions that can be seen in Fig. 6 (the spectra at 40 and 60 eV). Except for desogestrel the molecular ions were used as the parent ions. Low stability of the molecule of desogestrel thereby the low abundance of the appropriate molecular ion led to the use of the ion at m/z 293 as the parent ion. The parent to product ions transition m/z $293 \rightarrow 267$ was more sensitive than the usage of the transition m/z $311 \rightarrow 293$, 285, and 267. The most abundant product ions corresponded to the nonselective losses of the molecule of acetylene (EE2, GES, LNG and DES) or acetic acid (CPA). Attempt to achieve the adequate sensitivity using the more selective product ions originated in the cleavage of the steroidal ring failed.

In-source CID affected not only the MS abundance of the ions in the spectra but also the intensity of the MS signals. Increasing the fragmentor voltage value can enhance the intensity of the analytical signal for the selected compounds. The most intensive signals were obtained with the fragmentor energy 10 eV for all the selected compounds when the data were acquired in the ESI mode. In the APCI mode, the most intensive signals were obtained with the fragmentor energy 5 eV for all the compounds except for desogestrel (10 eV). The influence of the applied fragmentor voltage on the intensity of the MS signal is given in Fig. 7.

3.2.5. Analytical performance

The performance of the LC/MS method was evaluated by the determination of the linearity, sensitivity and repeatability of the method (see Table 3). Calibration curves were established from the injection of the standard solutions in the concentration range from 0.3 to 50 ng per injection for EE2, 0.6 to 50 ng for DES and 0.02 to 50 ng for GES, LNG and CPA for both the ESI and the APCI ionization modes. All calibration curves in both modes showed good linearity with regression coefficients higher than 0.99 even when the concentration ranges were quite wide.

The LOQs (S/N = 10) were determined from the 1 μl injections of the standard solution at the concentration of 600 pg μL^{-1} for EE2 and CPA when the data were acquired in the negative mode and at the concentrations of 20 pg μL^{-1} for GES, LNG and CPA (the positive mode) and 1200 pg μL^{-1} for DES. The LOQs determined from spiked water samples were slightly worse and they were estimated from 3 μl injections at the concentration of 100 pg μL^{-1} for EE2, 600 pg μL^{-1} for DES, 10 pg μL^{-1} for GES, LNG and CPA (the positive mode). The estimated LOQs were 310 pg for EE2, 15 pg for GES, 14.5 pg for LNG, 4.2 pg for CPA and 980 pg for DES (data acquired

Table 3
Chromatographic separations with the MS detection using the Zorbax SB-Phenyl column (100 mm × 2.1 mm i.d., 3.5 μm particle size), the data acquired in the selected ion monitoring (SIM) and selected reaction monitoring SRM modes

Compound	c (ng)[a]	r^2[b]	R.S.D.s (%)[c]	LOQ[d] (pg)		LOQ[e] (pg)	
				ESI	APCI	ESI	APCI
Ethynylestradiol	0.3–50	0.9991/0.9990	6.5/6.8[b]	300	280	420	410
Gestodene	0.02–50	0.9989/0.9989	8.4/7.9	16	14	50	38
Levonorgestrel	0.02–50	0.9987/0.9991	8.7/8.3	14	14	50	25
Cyproterone acetate	0.02–50	0.9975/0.9989	7.4/6.6	8/1000[f]	4/840[f]	16/1700[f]	14/156
Desogestrel	0.6–50	0.9958/0.9969	10.8/9.2	1100	960	1720	1760

Injected volume was 1 μL.
[a] Concentration range (in ng).
[b] Regression coefficients of the calibration curve for 10, 20, 30, 40 and 50 ng and the lowest concentration values, data for electrospray ionization/atmospheric pressure chemical ionization are given, respectively.
[c] Repeatability calculated from five replicates of standard solution at the concentrations 300 pg for EE2, 20 pg for GES, LNG, 20 pg for CPA, 600 pg for DES; data for electrospray ionization/atmospheric pressure chemical ionization are given, respectively.
[d] Limit of quantification equals to 10 s_{bl}, SIM mode.
[e] Limit of quantification equals to 10 s_{bl}, SRM mode.
[f] Data for positive/negative mode, respectively.

in APCI mode). Cyproterone acetate showed the MS signal in both the positive and the negative modes of ESI or APCI source, but LOQs for the negative mode were about 100 times higher than for the positive one. A higher background noise for ESI mode (its level was about three times higher than for the other compounds), perhaps resulting from the lower stability of desogestrel, was the main reason for its poor limit of quantification. The LOQs were slightly better for the APCI mode due to the lower level of the background noise. The low collision efficiency is the main reason for the slightly worse LOQs when the selected reaction monitoring was employed, but the great advantage of this mode is the reduction of the background noise.

The repeatability was evaluated from the injection of five replicates of the standard solution at various concentrations. The R.S.D.s in the range of 6.5–10.8% were found at the lowest concentration levels of the tested compounds. The best limits of quantification and the repeatabilities of the analyses were achieved using the atmospheric pressure chemical ionization source when the data were acquired using the time-scheduled selected ion monitoring mode in negative mode for ethynylestradiol and positive mode for the rest of the tested compounds. This configuration was used for all the following determinations of the estrogenic compounds in contraceptives and spiked water samples.

3.3. Estrogenic compounds in contraceptive products and water samples

The proposed LC/MS method was successfully applied to the analyses of ethynylestradiol together with gestodene, levonorgestrel, cyproterone acetate and desogestrel in four commonly used contraceptives. Although the relatively high contents of the estrogen and progestins in the contraceptives allowed their determination with the HPLC coupled to the UV detector, the simplicity of the drugs matrices is the best example for the initial test of the applicability of the proposed LC/MS method. The aqueous methanol or acetonitrile (50:50, v/v) appeared insufficient for the quantitative extraction of the analytes from the drug matrices. Approximately 30–40% of the declared amounts were found. On the contrary, the yield of the extraction with pure methanol or acetonitrile ranged from 97.5 to 99.87% (calculated from the difference of the declared and the found amounts). The repeatabilities of measurements were very good, only desogestrel demonstrated higher R.S.D.

Table 4
Declared and mean ($n = 5$) amounts of estrogens (in μg) found in the contraceptives

Products	Ethynylestradiol		Gestodene		Levonorgestrel		Cyproterone acetate		Desogestrel	
	D/F[a] (μg)	R[b] (%)	D/F[a] (μg)	R[b] (%)	D/F[a] (μg)	R[b] (%)	D/F[a] (μg)	R[b] (%)	D/F[a] (μg)	R[b] (%)
Minulet®[c]	30/29.4	98.0	75/73.5	98.0	–	–	–	–	–	–
Minisiston®[d]	30/29.5	98.3	–	–	125/123.8	99.0	–	–	–	–
Diana35®[e]	20/19.5	97.5	–	–	–	–	2000/1982	99.1	–	–
Mercilon®[f]	35/34.7	99.1	–	–	–	–	–	–	150/147.6	98.4

Injected volume was 1 μL.
[a] Declared/found amounts (in μg).
[b] Recovery (in %).
[c] R.S.D.s ($n = 5$) were 3.9% for ethynylestradiol and 3.6% for gestodene.
[d] R.S.D.s ($n = 5$) were 3.8% for ethynylestradiol and 2.8% for levonorgestrel.
[e] R.S.D.s ($n = 5$) were 3.6% for ethynylestradiol and 2.5% for cyproterone acetate.
[f] R.S.D.s ($n = 5$) were 3.6% for ethynylestradiol and 6.3% for desogestrel.

Table 5
Added and mean ($n = 5$) amounts of the estrogens found in the spiked water samples

Compound	Added (pg)	Upstream WWTP			Downstream WWTP		
		Found[a] (pg)	$R^{a,b}$ (%)	R.S.D.s[c] (%)	Found[c] (pg)	$R^{a,b}$ (%)	R.S.D.s[c] (%)
Ethynylestradiol	300	296	98.7	8.9	295	98.3	9.1
Gestodene	30	28	93.3	7.7	29	96.7	7.4
Levonorgestrel	30	29	96.7	8.1	28	93.3	7.5
Cyproterone acetate	30	29	96.7	6.9	29	96.7	7.7
Desogestrel	600	591	98.5	9.5	593	98.8	10.1
Compound	Added (pg)	Tap water			Brno dam		
		Found[a] (pg)	$R^{a,b}$ (%)	R.S.D.s[c] (%)	Found[c] (pg)	$R^{a,b}$ (%)	R.S.D.s[c] (%)
Ethynylestradiol	300	298	99.3	7.1	296	98.7	9.3
Gestodene	30	29	96.7	7.9	29	96.7	7.5
Levonorgestrel	30	29	96.7	8.2	30	100	7.7
Cyproterone acetate	30	28	93.3	6.6	28	93.3	7.1
Desogestrel	600	595	99.2	9.4	590	98.3	10.5

Injected volume was 3 μL.
[a] Means calculated for five samples.
[b] Recovery (in %).
[c] R.S.D.s calculated from five replicates of each of the five samples.

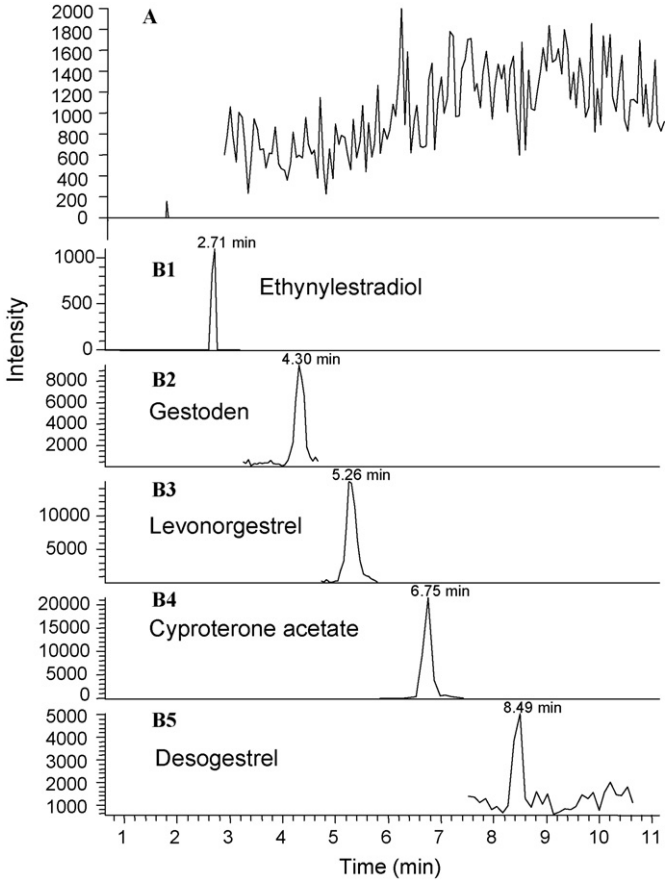

Fig. 8. The chromatogram of the HPLC/APCI-IT-MS analysis of the waste water sample (A) and the same waste water sample enriched at 150 pg μL^{-1} with ethynylestradiol (B1), 500 pg μL^{-1} with desogestrel (B5), 10 pg μL^{-1} with cyproterone acetate (B4), 20 pg μL^{-1} with gestodene (B2), levonorgestrel (B3). The injection volume was 1 μL.

at 6.3%. The results of the appropriate analyses are given in Table 4.

Water samples represent more complicated matrices for estrogens determination than contraceptives. Low contents of estrogenic compounds in the environmental samples (ng–pg L^{-1}) require application of an extraction step (mostly solid-phase extraction) prior to the final by adding defined amounts of the tested estrogens to the river water samples. All water samples were also analyzed prior to the addition of standards and none of them showed presence of the tested compounds. Fig. 8 shows a chromatogram of fresh waste water (A) and the same sample spiked with ethynylestradiol at 150 pg μL^{-1}, with desogestrel at 500 pg μL^{-1}, with cyproterone acetate at 10 pg μL^{-1} and with gestodene and levonorgestrel at 20 pg μL^{-1} each (B). Results of the analyses of the spiked water samples are presented in Table 5. Since the complicated sample preparation was not applied, all the analyses showed very good R.S.D.s being close to the values found when analyzing the standard solutions. The analyses of samples with higher contents of added estrogenic compounds showed similar difference between the added and the found amounts (in the range from 1 to 4%) with corresponding R.S.D.s in the range from 4.5 to 7.4%.

4. Conclusion

The quick and highly sensitive liquid chromatographic/ion-trap mass spectrometric method for the separation and simultaneous quantification of ethynylestradiol, gestodene, levonorgestrel, cyproterone acetate and desogestrel was developed and optimized using a Zorbax SB-Phenyl column and aqueous methanol as the mobile phase. Excellent quantification limits were obtained using the LC/MS in SIM or SRM modes. The repeatability of determination was better than 11%. The on-column limits of quantification (10 S/N) of 300 pg of EE2,

Table 6
Comparison of characteristics of the liquid chromatographic methods for determination of estrogens

Analyte	Sample matrix	Sample preparation	Detection	LOD	Ref.
EE2[a], LNG[b]	–	–	GC/MS ESI[c]/APCI[d]-MS ESI/APCI-MS/MS	10, 20 ng mL^{-1} 10, nd; 0.4, 10 ng mL^{-1} 2, nd; 10, 40 ng mL^{-1}	[5]
EE2	Human plasma	Centrifuged, derivatization with dansyl chloride	ESI-MS/MS (QqQ)[e]	2.5 pg mL^{-1}	[11]
CPA[f]	Human plasma	On-line clean-up using restricted access material	ESI-MS/MS (QqQ)	120 pg mL^{-1}	[12]
EE2, LNG	Water	SPE	UV-APCI/ESI-MS	UV (242 nm) 50, 50 ng L^{-1} ESI 100, 2 ng L^{-1} APCI 3000, 20 ng L^{-1}	[14]
EE2	Water	SPE	IT[g]-MS	2 ng L^{-1}	[15]
EE2, LNG	Sediment	PSE, clean-up using restricted access material	ESI-MS	5, 0.5 ng g^{-1}	[16]
EE2	Water	SPE	ESI-MS/MS (QqQ)	2 ng L^{-1}	[21]
EE2, GES[h]	Contraceptives	–	UV (220 nm)	0.8, 2.3 ng mL^{-1}	[22]

[a] Ethynylestradiol.
[b] Levonorgestrel.
[c] Electrospray.
[d] Atmospheric pressure chemical ionization.
[e] Triple quadrupole.
[f] Cyproterone acetate.
[g] Ion trap, SPE: solid-phase extraction, PSE: pressurized solvent extraction.
[h] Gestodene.

14 pg of GES, LNG, 4 pg of CPA and 960 pg of DES per injection (1 μL), respectively, allow the application of the proposed method for the studies of these estrogenic substances in biological materials as well as for determination of these potentially hazardous compounds in environmental samples. The lifetime of the analytical column was estimated to be 300–500 injections since no significant difference in RT was observed after 6 months. The stability of MS signal was constant at least 2 weeks. Then the cleaning of the MS source was needed. Future investigations will lead to development of isolation, pre-concentration and pre-separation procedures for the determination of these compounds in environmental samples as wastewater, sediments, soils, etc.

A comparison with the literature is difficult or practically impossible since no data were found in literature for more complicated mixtures of the estrogens (except of one compound in the presence of EE2 using UV and MS detection for their determination in tablets and plasma, respectively). No quantitative data were found for desogestrel. Only levonorgestrel with ethynylestradiol was from time to time identified and quantified in environment (see Table 6). Sensitivity of proposed method is lower (ca. 20-times for EE2) or comparable and/or better for other compounds compare to tripleQ. Sensitivity of ion-trap MS is lower compare to tripleQ (as it is well-known); 200 pg in negative mode for EE2 is ca. 20-times higher; 10 and 4 pg for GES, CPA, LNG are comparable, no data for DES were found. In some cases pre-concentration/pre-separation procedure(s) are used and results are not presented in absolute values thus the comparison of LODs is not relevant.

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References

[1] M. Kuster, M.J. Lopez de Alda, D. Barcelo, Trends Anal. Chem. 23 (2004) 790.
[2] F. Ingerslev, B. Halling-Sorensen, Danish Environmental Protection Agency, Working Report No. 44, 2003.
[3] M.J. Lopez de Alda, D. Barcelo, Fresenius J. Anal. Chem 371 (2001) 437.
[4] R.W. Giese, J. Chromatogr. A 1000 (2003) 401.
[5] M.S. Diaz-Cruz, M.J. Lopez de Alda, R. Lopez, D. Barcelo, J. Mass Spectrom. 38 (2003) 917.
[6] T. Isobe, H. Shiraishi, M. Yasuda, A. Shinoda, H. Suzuki, M. Morita, J. Chromatogr. A 984 (2003) 195.
[7] L. Brossa, E. Pocurull, F. Borrul, R.M. Marce, Chromatographia 59 (2004) 419.
[8] S. Zuehlke, U. Duennbier, T. Heberer, J. Sep. Sci. 28 (2005) 52.
[9] S. Reddy, C.R. Iden, B.J. Brownawell, Anal. Chem. 77 (2005) 7032.
[10] H.B. Theron, C. Coetzee, F.C.W. Sutherland, J.L. Wiesner, K.J. Swart, J. Chromatogr. B 813 (2004) 331.
[11] W. Li, Y. Li, A.C. Li, S. Zjou, W. Naidong, J. Chromatogr. B 828 (2005) 223.
[12] B. Christiaens, M. Fillet, P. Chiap, O. Rheida, A. Ceccato, B. Streel, J. De Graeve, J. Crommen, Ph. Hubert, J. Chromatogr. A 1056 (2004) 105.
[13] M.R. Anari, R. Bakhtiar, B. Zhu, S. Huskey, R.B. Franklin, D.C. Evans, Anal. Chem. 74 (2002) 4133.
[14] M.J. Lopez de Alda, D. Barcelo, J. Chromatogr. A 892 (2000) 391.
[15] V. Ingrand, G. Herry, J. Beausse, M.R. de Roubin, J. Chromatogr. A 1020 (2003) 99.

[16] M. Petrovic, S. Tavazzi, D. Barcelo, J. Chromatogr. A 971 (2002) 37.
[17] F. Zhang, M.J. Bartels, J.C. Brodeur, E.L. McClymont, K.B. Woodburn, Rapid Commun. Mass Spectrom. 18 (2004) 2739.
[18] K. Gallicano, I. McGilveray, J. Clin. Ligand Assay 20 (1997) 10.
[19] M. Kranendijk, J.C.M. Waterval, G.W. Somsen, G.J. de Jong, J. Sep. Sci 28 (2005) 1796.
[20] A.S. Pereira, G.D. Mendes, L.S.O.B. Oliveira, H.F. Valle, G. De Nucci, J. Chromatogr. Sci. 43 (2005) 513.
[21] M. Farré, R. Brix, M. Kuster, F. Rubio, Y. Goda, M.J. Lopez de Alda, D. Barcelo, Anal. Bioanal. Chem. 385 (2006) 1001.
[22] A. Laban, S. Markovic, M. Stankov, P. Djurdjevic, Anal. Lett. 37 (2004) 273.